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Dysfunctional HDL: from structure-function-relationships to biomarkers

Riwanto, Meliana ; Rohrer, Lucia ; von Eckardstein, Arnold ; Landmesser, Ulf

Abstract: Reduced plasma levels of HDL-C are associated with an increased risk of CAD and myocardial infarction, as shown in various prospective population studies. However, recent clinical trials on lipid-modifying drugs that increase plasma levels of HDL-C have not shown significant clinical benefit. Notably, in some recent clinical studies, there is no clear association of higher HDL-C levels with a reduced risk of cardiovascular events observed in patients with existing CAD. These observations have prompted researchers to shift from a cholesterol-centric view of HDL towards assessing the function and composition of HDL particles. Of importance, experimental and translational studies have further demonstrated various potential antiatherogenic effects of HDL. HDL has been proposed to promote macrophage reverse cholesterol transport and to protect endothelial cell functions by prevention of oxidation of LDL and its adverse endothelial effects. Furthermore, HDL from healthy subjects can directly stimulate endothelial cell production of nitric oxide and exert anti-inflammatory and antiapoptotic effects. Of note, increasing evidence suggests that the vascular effects of HDL can be highly heterogeneous and HDL may lose important anti-atherosclerotic properties and turn dysfunctional in patients with chronic inflammatory disorders. A greater understanding of mechanisms of action of HDL and its altered vascular effects is therefore critical within the context of HDL-targeted therapies.

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Dysfunctional HDL: From Structure-Function-Relationships to Biomarkers

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and Ulf Landmesser

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Abstract

Reduced plasma levels of HDL-C are associated with an increased risk of CAD and myocardial infarction, as shown in various prospective population studies. However, recent clinical trials on lipid-modifying drugs that increase plasma levels of HDL-C have not shown significant clinical benefit. Notably, in some recent clinical studies, there is no clear association of higher HDL-C levels with a reduced risk of cardiovascular events observed in patients with existing CAD. These observations have prompted researchers to shift from a cholesterol-centric view of HDL towards assessing the function and composition of HDL particles.

Of importance, experimental and translational studies have further demonstrated various potential antiatherogenic effects of HDL. HDL has been proposed to promote macrophage reverse cholesterol transport and to protect endothelial cell functions by prevention of oxidation of LDL and its adverse endothelial effects. Furthermore, HDL from healthy subjects can directly stimulate endothelial cell production of nitric oxide and exert anti-inflammatory and antiapoptotic effects. Of note, increasing evidence suggests that the vascular effects of HDL can be highly heterogeneous and HDL may lose important anti-atherosclerotic properties and turn dysfunctional in patients with chronic inflammatory disorders. A greater understanding of mechanisms of action of HDL and its altered vascular effects is therefore critical within the context of HDL-targeted therapies.

Keywords

HDL dysfunction • Endothelial function • Atherosclerosis • CAD

Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACS	Acute coronary syndrome
apoA-I	Apolipoprotein A1
apoA-II	Apolipoprotein A2
apoB	Apolipoprotein B
apoA-IV	Apolipoprotein A-IV
apoC-III	Apolipoprotein C-III
apoE	Apolipoprotein E
apoJ	Apolipoprotein J
Bad	Bcl-2-associated death promoter
Bcl-xL	B-cell lymphoma-extra large
Bid	BH3 interacting domain death agonist

CAD	Coronary artery disease
caspase	Cysteine-aspartic proteases
CETP	Cholesteryl ester transfer protein
CHF	Chronic heart failure
CKD	Chronic kidney disease
eNOS	Endothelial nitric oxide synthase
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HPLC	High-performance liquid chromatography
ICAM-1	Intercellular Adhesion Molecule 1
LCAT	Lecithin cholesterol acyltransferase
MS/MS	Tandem mass spectrometry
LDL	Low density lipoprotein
L-NAME	L-NG-Nitroarginine Methyl Ester
LOX-1	Lectin-type oxidized LDL receptor 1
LpPLA2	Lipoprotein-associated phospholipase A2
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MDA	Malondialdehyde
MI	Myocardial infarction
MPO	Myeloperoxidase
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
oxLDL	Oxidized low density lipoprotein
PAF-AH	Platelet-activating factor acetylhydrolase
PI3K	Phosphoinositide-3-kinase
PLTP	Phospholipid transport protein
PON1	Paraoxonase 1
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
RCT	Reverse cholesterol transport
rHDL	Reconstituted high-density lipoprotein
S1P	Sphingosine-1-phosphate
SAA	Serum amyloid A
SNP	Single nucleotide polymorphism
sPLA ₂ -IIa	Secretory phospholipase A ₂ -IIa
SR-BI	Scavenger receptor type B1
TNF- α	Tumor necrosis factor-alpha
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low density lipoprotein

1 Introduction

It was shown in multiple large prospective studies of cardiovascular risk factors that reduced plasma levels of HDL-cholesterol (HDL-C) are associated with an increased risk of coronary artery disease (CAD) (Castelli et al. 1986; Cullen et al. 1997; Di Angelantonio et al. 2009; Gordon et al. 1977; Sharrett et al. 2001). Multiple biological functions of HDL have been identified, whereby HDL may exert antiatherogenic effects (Annema and von Eckardstein 2013; Barter et al. 2004; Mineo et al. 2006; Rader 2006; Riwanto and Landmesser 2013), e.g., HDL from healthy subjects has been shown to directly promote endothelial antiapoptotic, anti-inflammatory, and antithrombotic effects (Mineo et al. 2006; Nofer et al. 2004; Rye and Barter 2008; Tall et al. 2008; Yuhanna et al. 2001). Accordingly, interventions to improve HDL-C levels and/or HDL function are being intensely evaluated as a potential therapeutic strategy to reduce cardiovascular risk. However, increasing evidence suggests that the endothelial and vascular effects of HDL are highly heterogeneous and vasoprotective properties of HDL are impaired in patients with diabetes, CAD, or chronic kidney dysfunction (Besler et al. 2011; Khera et al. 2011; Riwanto et al. 2013; Sorrentino et al. 2010).

Intriguingly, several recent clinical trials testing the effects of HDL-C-raising therapies have failed to demonstrate cardiovascular risk reduction in patients with CAD. The Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial testing the impact of the CETP inhibitor torcetrapib on clinical outcome showed increased risk of mortality and morbidity in patients at high risk for coronary events, despite a substantial increase of HDL-C levels (Barter et al. 2007). Dalcetrapib, another CETP inhibitor, modestly increased HDL-C levels, but the phase 3 trial dal-OUTCOMES study was terminated before completion to a lack of efficacy (Schwartz et al. 2012). More recently, the HPS2-THRIVE trial results showed that adding extended-release niacin/laropiprant, another HDL-cholesterol-raising agent, to statins did not reduce the risk of cardiovascular event (Haynes et al. 2013). Taken together, these observations strongly suggest that plasma HDL-C levels per se are not an optimal therapeutic target.

Of note, accumulating evidence suggests that the vascular effects of HDL can be highly heterogeneous and may turn dysfunctional. HDL loses potential anti-atherosclerotic properties in patients with chronic inflammatory disorders, such as the antiphospholipid syndrome (Charakida et al. 2009), systemic lupus erythematosus and rheumatoid arthritis (McMahon et al. 2006), scleroderma (Weihrach et al. 2007), metabolic syndrome (de Souza et al. 2008), diabetes (Persegol et al. 2006; Sorrentino et al. 2010), and CAD (Ansell et al. 2003; Besler et al. 2011; Riwanto et al. 2013). In a study of 189 patients with chronic kidney disease on hemodialysis, an impaired anti-inflammatory capacity of HDL was correlated with a poor clinical outcome (Kalantar-Zadeh et al. 2007). Furthermore, HDL isolated from subjects with type 1 or type 2 diabetes mellitus or abdominal obesity had reduced capacity to reverse the inhibition of aortic ring endothelium-dependent relaxation by oxLDL as compared to HDL from healthy control subjects

(Persegol et al. 2006, 2007). Importantly, the heterogeneity of the vascular effects of HDL may be attributed to changes in the HDL-associated proteome and lipidome, i.e., changes in the amount and type of proteins and lipids bound to the HDL particle and also posttranslational modifications. In particular, HDL is known to be susceptible to modification *in vitro* by a variety of oxidants, such as metal ions, peroxy and hydroxyl radicals, aldehydes, various myeloperoxidase (MPO)-generated oxidants, lipoxygenase, phospholipase A2, elastase, nonenzymatic glycation, and homocysteinylation (Ferretti et al. 2006). These oxidative modifications may contribute to the generation of dysfunctional proinflammatory HDL.

2 HDL and Reverse Cholesterol Transport

2.1 Mechanisms Under Physiological Conditions

One of the antiatherogenic effects of HDL has been attributed to its function in macrophage reverse cholesterol transport (RCT), i.e., the removal of excess cholesterol from lipid-laden macrophage foam cells in the atherosclerotic plaque and its transport to the liver for excretion in the bile (Rader 2006). The first step of macrophage RCT involves the hydrolysis of cytoplasmic cholesteryl ester into free cholesterol followed by the efflux of the free cholesterol to mature HDL or extracellular lipid-poor apoA-I. Macrophage cholesterol efflux is mediated by active transport systems, which include the ATP-binding cassette transporters ABCA1 and ABCG1 (Rader 2006; Tall et al. 2008). Studies in macrophages from ABCA1-knockout or ABCA1-overexpressing mice have shown that ABCA1 primarily mediates the cholesterol efflux to lipid-poor apoA-I (Bortnick et al. 2000; Haghighpassand et al. 2001). In contrast, ABCG1 largely mediates cholesterol efflux from macrophages to mature HDL (Tall et al. 2008).

A study by Wang et al. quantitatively assessed the roles of SR-BI, ABCA1, and ABCG1 in macrophage RCT in mice *in vivo* (Wang et al. 2007a). Using primary macrophages lacking SR-BI, the authors demonstrated that SR-BI did not promote macrophage RCT *in vivo* after intraperitoneal injection. In contrast, both ABCA1 and ABCG1 contributed to macrophage RCT *in vivo*. The study also demonstrated that transplantation of bone marrow from ABCA1/ABCG1-deficient mice accelerated atherosclerotic lesion formation in LDL receptor-deficient mice (Wang et al. 2007a). Interestingly, Yvan-Charvet et al. also demonstrated that SR-BI failed to stimulate net cholesterol efflux from HEK293 cells to plasma HDL and inhibited ABCG1-mediated cholesterol efflux, which was at least in part due to the increased uptake of HDL cholesteryl esters into these cells (Yvan-Charvet et al. 2008).

HDL-associated cholesterol is subsequently esterified by LCAT which transfers a fatty acyl residue from phospholipids to the 3- β -hydroxy group of cholesterol. The final steps of RCT involve the uptake of HDL-C by the liver and its excretion in the bile. Cholesterol esters and free cholesterol in HDL can either be directly taken

up by the liver via SR-BI or transferred to apoB containing lipoproteins in a process mediated by CETP (Cuchel et al. 2009). In the latter pathway, cholesterol esters are taken up by the liver via the LDL receptor and then hydrolyzed to free cholesterol which can be excreted directly into the bile or following conversion to bile acid.

2.2 Alterations of the Cholesterol Efflux Capacity of HDL in Cardiovascular Disease

A case-control study by Khera et al. showed that the cholesterol efflux capacity of apoB-depleted serum was inversely related to carotid IMT in healthy volunteers and to the likelihood of angiographic CAD, even after adjustment for HDL-C and apoA-I levels (Khera et al. 2011). In a separate study, enhanced cholesterol efflux activity from ABCA1-stimulated macrophages was associated with reduced risk of prevalent CAD in unadjusted models in two case-control cohorts: an angiographic cohort comprising stable subjects undergoing elective diagnostic coronary angiography and an outpatient cohort (Li et al. 2013). However, the inverse risk relationship remained significant after adjustment for traditional CAD risk factors only within the outpatient cohort (Li et al. 2013). Surprisingly, higher cholesterol efflux activity was associated with an increase in prospective 3-year risk of myocardial infarction/stroke and major adverse cardiovascular events. The authors further observed that the HDL fraction ($1.063 < d < 1.21$) contained only a minority ($\approx 40\%$) of [(14)C] cholesterol released, with the majority found within the lipoprotein particle-depleted fraction, where $\approx 60\%$ was recovered after apoA-I immunoprecipitation. This is in agreement with previous findings on plasmas from patients with apoA-I deficiency, such as Tangier disease and LCAT deficiency, whereby the cholesterol efflux capacity amounted to at least 40 and 20 % of total and apoB-depleted plasmas from healthy controls, respectively (von Eckardstein et al. 1995a). However, in these studies the considerable residual cholesterol efflux capacity of HDL-free or HDL-poor plasma was explained by the abundance of apoA-I-free HDL particles containing apoA-IV, apoA-II, or apoE rather than by albumin (Huang et al. 1994, 1995; von Eckardstein et al. 1995a, b).

Importantly, impaired cholesterol efflux capacity of HDL has been associated with structural changes of the HDL components. Two groups independently demonstrated that oxidative modification of apoA-I, particularly by oxidation of methionine, tyrosine, or tryptophan residues through the myeloperoxidase pathway, dramatically reduced the ability of apoA-I to promote cholesterol efflux through the ABCA1 pathway (Bergt et al. 2004; Huang et al. 2014; Shao et al. 2006, 2010). Both groups also demonstrated that apoA-I in patients with cardiovascular disease was oxidatively modified and defective in promoting cholesterol efflux from macrophages via ABCA1 (Bergt et al. 2004; Shao et al. 2012; Zheng et al. 2004).

Of note, the cholesterol efflux capacity as determined by the assay protocol of Khera et al. which is widely used after its prominent publications is potentially confounded by several HDL-independent factors: the 4-h long incubation with cells raises the possibility that serum components deregulate the activity of ABCA1 and

ABCG1. For example, free fatty acids and cytokines are known to alter the expression of ABCA1 and ABCG1 on both the transcriptional and posttranslational level. The use of serum instead of plasma implies the activation of thrombin and other proteases which are known to degrade prebeta-HDL (Eckardstein 2012).

3 Effects of HDL on LDL Oxidation

3.1 Mechanisms Under Physiological Conditions

The antioxidative properties of HDL were first reported by Bowry (Bowry et al. 1992). It has been suggested that HDL limits the formation of oxidized LDL (oxLDL) and oxidation of LDL has been proposed for decades to be a key event in atherogenesis (Heinecke 1998; Witztum and Steinberg 1991). LDL accumulates in the subendothelial space where it is oxidized by different pathways such as lipoxygenase, myeloperoxidase, or NADPH oxidase pathways (Diaz et al. 1997). OxLDL induces the expression of various proinflammatory cytokines, chemokines, and adhesion molecules (Navab et al. 1996). In addition, it promotes monocyte chemotaxis, their differentiation into macrophages, and the subsequent oxLDL uptake that convert them into foam cells, a hallmark of atherosclerotic plaques (Chisolm et al. 1999).

HDL is a major carrier of lipid oxidation products and specifically apoA-I, the main protein constituent of HDL, binds to and removes lipid hydroperoxides of LDL in vitro and in vivo (Navab et al. 2000b). In addition, apoA-I may directly reduce cholesteryl ester hydroperoxides and phosphatidylcholine hydroperoxides by oxidation of specific Met residues in the protein (Garner et al. 1998). Interestingly, the resulting oxidized HDL is more rapidly and selectively removed by hepatocytes than native HDL (Garner et al. 1998). Treatment of human artery wall cells with apoA-I but not apoA-II, or by addition of native HDL, apoA-I peptide mimetics, or paraoxonase, prevents the cells from oxidizing LDL in vitro (Navab et al. 2000a). The antioxidative function of apoA-I was corroborated by the finding that recombinant HDL containing only apoA-I and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) was as effective as native HDL in preventing LDL oxidation. In addition, in vivo studies have demonstrated that apoA-I can act as an antioxidative, anti-inflammatory, and anti-atherosclerotic agent (Nicholls et al. 2005a, b; Paszty et al. 1994). This further underlines a key antioxidant role for the HDL major protein apoA-I (Zerrad-Saadi et al. 2009).

Several other HDL-associated apolipoproteins have also been shown to contribute to its antioxidant effects. ApoE has been demonstrated to show isoform-dependent antioxidant activity (Miyata and Smith 1996). ApoE2 stimulates endothelial NO release and exerts anti-inflammatory effects (Sacre et al. 2003). In contrast, apoE4 has been suggested to be proinflammatory (Ophir et al. 2005). Another HDL-associated protein, apoA-IV, showed anti-atherosclerotic, anti-inflammatory, and antioxidant actions in vivo (Ostos et al. 2001; Recalde

et al. 2004; Vowinkel et al. 2004). In addition, apoJ, also called clusterin, has been reported to block LDL oxidation by artery wall cells (Navab et al. 1997).

Furthermore, ApoA-II-enriched HDL isolated from transgenic human apoA-II mice protected VLDL from oxidation more efficiently than control HDL (Boisfer et al. 2002). In contrast, in other studies, dyslipidemic mice overexpressing human apoA-II showed accelerated atherosclerosis and reduced antioxidative activity of HDL, which may be attributed to the displacement of apoA-I and PON1 by apoA-II in HDL particles (Ribas et al. 2004; Rotllan et al. 2005). Notably, in apparently healthy subjects from the prospective EPIC-Norfolk (European Prospective Investigation into Cancer and Nutrition-Norfolk) cohort, apoA-II was found to be associated with a decreased risk of future CAD in a nested case-control study (Birjmohun et al. 2007).

Importantly, HDL carries antioxidant enzymes that have the capacity to prevent lipid oxidation or degrade lipid hydroperoxides such as serum paraoxonase/arylesterase 1 (PON1), lecithin/cholesterol acyltransferase (LCAT), and platelet-activating factor acetylhydrolase (PAF-AH). Specifically PON1 has been suggested to be an important regulator of the potential antiatherogenic capacity of HDL (Shih et al. 1998; Tward et al. 2002). It is secreted from the liver and associates with HDL in the plasma. Many studies revealed that PON1 degrades oxidized proinflammatory lipids, measured as a reduction in lipid peroxides (Mackness et al. 1991; Shih et al. 2000; Watson et al. 1995). Higher PON1 activity has been reported to be associated with a lower incidence of major cardiovascular events in human. Conversely, reduced activity of PON1 has been associated with pathological conditions such as diabetes, chronic renal failure rheumatoid arthritis, and various dementia (Soran et al. 2009).

LCAT is another HDL-associated enzyme with antioxidative properties. The antioxidant role of LCAT is thought to be its capacity to hydrolyze oxidized acyl chains from phosphatidylcholine-based oxidized phospholipids and oxidized free fatty acids (Goyal et al. 1997; Subramanian et al. 1999). Genetics and biochemical characterizations of HDLs from patients with low HDL disorders demonstrated that HDL-LCAT activity was reduced in all LCAT mutation carriers as well as in patients with HDL deficiency due to mutation in APOA1 or ABCA1 (Soran et al. 2009; von Eckardstein et al. 1995a). In vivo study in mice deficient for LDL receptor and leptin revealed that LCAT overexpression decreased autoantibodies to oxLDL (Mertens et al. 2003). Besides LCAT and PON1, PAF-AH, another HDL-associated enzyme, nowadays also known as lipoprotein-associated phospholipase A2 (LpPLA2), is able to hydrolyze oxidized phospholipids (Marathe et al. 2003; Noto et al. 2003). The local expression of PAF-AH in arteries of non-hyperlipidemic rabbits reduced the accumulation of oxidatively modified LDL without changing plasma levels of PAF-AH and reduced the expression of endothelial cell adhesion molecules (Arakawa et al. 2005). PAF-AH deficiency caused by a missense mutation in the gene has been indicated to be an independent risk factor for CAD in Japanese men (McIntyre et al. 2009). In contrast to this both activity and mass concentration of LpPLA2 in total serum has been associated with increased rather than decreased cardiovascular risk (Lp et al. 2010). However, recently HDL-associated LpPLA2 was found to be

inversely related to risk of coronary events in stable CAD patients (Rallidis et al. 2012). Hence, the lipoprotein distribution of LpPLA2 may determine the pro- or antiatherogenic role of PAF-AH/LpPLA2. Furthermore, plasma levels of PAF-AH are also shown to be an independent risk marker of CAD (Garza et al. 2007). However, a study by Holleboom et al. showed that the reduction in LCAT and PAF-AH activities due to LCAT mutations was not associated with increased plasma lipid peroxidation (Holleboom et al. 2012).

3.2 Impairment of the Anti-Oxidative Effects of HDL in Patients After Surgery and With Cardiovascular Disease

In the acute phase response to surgery, van Lenten et al. demonstrated that the serum amyloid A (SAA) levels in HDL was increased and the activities of PON1 and PAF-AH were reduced (Van Lenten et al. 1995). Concomitant with these changes the anti-inflammatory capacity of HDL was lost in both humans and rabbits (Van Lenten et al. 1995). Isolated HDL from patients before and immediately after surgery were compared for the effects of HDL on LDL-induced monocyte transmigration and lipid hydroperoxide formation (Van Lenten et al. 1995). Prior to surgery, HDL completely inhibited the LDL-induced increase in monocyte transmigration and lipid hydroperoxide formation. In marked contrast, the “acute phase” HDL obtained from the same patients 2–3 days after surgery revealed a significant LDL-induced monocyte transmigration and was less effective in inhibiting lipid hydroperoxide formation. In other words, HDL in the same patient had been transformed from anti-inflammatory towards proinflammatory particles (Van Lenten et al. 1995). Similarly findings were reported in mice infected with influenza A or *Chlamydia pneumoniae*. HDL isolated from virus-infected wild-type mice had impaired capacity to block LDL oxidation as well as LDL-induced monocyte chemotactic activity in human artery wall cell co-cultures (Van Lenten et al. 2001). The mice infected with *C. pneumoniae* had decreased PON1 activity and reduced HDL capacity to prevent LDL oxidation at days 2 and 3 postinfection (Campbell et al. 2010). Of note, studies by Navab et al. showed that HDL isolated from patients with CAD failed to prevent LDL oxidation by human artery wall cells (Navab et al. 2000a) and had impaired capacity to inhibit LDL-induced monocyte chemotactic activity (Navab et al. 2001b). Similar results were obtained with HDL isolated from mice genetically predisposed to diet-induced atherosclerosis; it became proinflammatory when the mice were fed an atherogenic diet (Navab et al. 2001a). A subsequent small study by Ansell et al. suggested that the capacity of HDL to alter LDL-induced monocyte chemotactic activity in patients with CAD was somewhat improved after 6 weeks of simvastatin therapy (Ansell et al. 2003). However, HDL from patients with CAD on statin therapy remained proinflammatory when compared to HDL from healthy subjects, despite a significant improvement in plasma lipid levels.

Interestingly, impaired antioxidant properties and increased oxidized fatty acids content were found in HDL from patients with type 2 diabetes (Morgantini

et al. 2011). The authors speculated that the elevated levels of oxidized fatty acids in HDL from these patients may account for the impaired antioxidant properties of the lipoprotein (Morgantini et al. 2011). Moreover, in a different study, the ability of HDL to prevent LDL oxidation was found to be reduced in patients with acute coronary syndrome but not in patients with stable coronary artery syndrome (Patel et al. 2011). The antioxidative and cholesterol efflux capacities of HDL were also found to be reduced in ischemic cardiomyopathy (Patel et al. 2013).

4 Effects of HDL on Endothelial Nitric Oxide Bioavailability

4.1 Mechanisms Under Physiological Conditions

Endothelial nitric oxide plays a crucial role in the regulation of vascular tone, platelet aggregation, and angiogenesis. Endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) has been shown to exert a variety of atheroprotective effects in the vasculature, such as anti-inflammatory and antithrombotic effects (Landmesser et al. 2004). Consequently, reduced endothelial NO bioavailability has been proposed to promote initiation and progression of atherosclerosis (Landmesser et al. 2004).

It was shown that HDL can directly stimulate eNOS-mediated NO production (Yuhanna et al. 2001). There have been several studies that consistently demonstrated the capacity of HDL to modulate eNOS expression and to stimulate endothelial NO production in vitro and in vivo (Besler et al. 2011; Kuvin et al. 2002; Mineo et al. 2003; Nofer et al. 2004; Ramet et al. 2003; Sorrentino et al. 2010). Furthermore, administration of reconstituted HDL (rHDL) has been shown to improve endothelial function in patients with hypercholesterolemia or in subjects with isolated low HDL due to heterozygous loss-of-function mutations in the ABCA1 gene locus (Bisoendial et al. 2003; Spieker et al. 2002). Several mechanisms have been proposed to account for the endothelial NO-stimulating capacity of HDL. Early studies have demonstrated that HDL prevents oxLDL-mediated eNOS displacements from caveolae and restores enzyme stimulation (Uittenbogaard et al. 2000). Yuhanna et al. demonstrated that HDL binding to endothelial SR-BI is required for eNOS activation (Yuhanna et al. 2001). This binding activates the phosphatidylinositol-3-kinase/Akt signaling pathway and the MAP kinase/extracellular signal-regulated kinase pathway (Mineo et al. 2003). Activation of endothelial Akt by HDL stimulates phosphorylation of eNOS at serine residue 1177 (Mineo et al. 2003; Nofer et al. 2004), which is known to be an important regulatory mechanism leading to eNOS activation (Dimmeler et al. 1999).

There are many different components of HDL known to play a role in the endothelial NO-stimulating capacity. The potential interaction of apoA-I with eNOS in cultured endothelial cells has been previously reported (Ramet et al. 2003). However, lipid-free apoA-I failed to activate eNOS despite being the ligand for SR-BI, suggesting that other HDL components may be important or are

required to support the conformation of apoA-I to allow its interaction with SR-BI and to stimulate eNOS (de Beer et al. 2001). In isolated endothelial cell plasma membranes, anti-apoA-I antibody blocks eNOS activation by HDL in vitro (Yuhanna et al. 2001). In contrast anti-apoA-II antibody further enhances eNOS stimulation by HDL (Yuhanna et al. 2001). Furthermore, lysophospholipids may play a role in eNOS activation as demonstrated in several studies. A study by Nofer et al. suggested that HDL-associated sphingolipids such as sphingosylphosphorylcholine, sphingosine-1-phosphate, and lysosulfatide caused eNOS-dependent relaxation of precontracted aortic rings from mice by binding to the lysophospholipid receptor S1P3 expressed in endothelial cells (Nofer et al. 2004). However, the vasodilatory response of HDL was not completely abolished in S1P3-deficient mice (Nofer et al. 2004).

Recently, we demonstrated that the HDL-associated enzyme PON1 is participating in HDL's capacity to stimulate endothelial NO production and to exert NO-dependent endothelial atheroprotective effects (Besler et al. 2011). Inhibition of PON1 in HDL from healthy subjects impaired the capacity of HDL to stimulate endothelial NO production in human aortic endothelial cells. In addition HDL isolated from PON1-deficient mice failed to stimulate NO production in mouse aortic endothelial cells (Besler et al. 2011). Furthermore, inhibition of eNOS-mediated NO production by the pharmacological inhibitor L-NAME prevented the inhibitory effects of HDL from healthy subjects on nuclear factor κ B (NF- κ B) activity, vascular cell adhesion molecule (VCAM)-1 expression, and endothelial monocyte adhesion. These clearly indicate that the capacity of HDL to stimulate endothelial NO production is important for these endothelial anti-inflammatory effects of HDL (Besler et al. 2011).

4.2 Impaired HDL Capacity to Stimulate NO Production in Patients with Cardiovascular Disease

We and others have recently shown that HDL isolated from patients with stable CAD, diabetes, chronic kidney diseases (CKD), or acute coronary artery syndrome (ACS) displays altered endothelial effects when compared to HDL from healthy subjects. Of note, HDL from patients with diabetes in contrast to HDL from healthy subjects failed to stimulate endothelial cell NO production and to promote endothelial repair in a carotid artery injury model in mice (Sorrentino et al. 2010). Moreover, HDL from patients with either stable CAD or an ACS, in contrast to HDL from age- and gender-matched healthy subjects, inhibited endothelial cell NO production and lost the capacity to limit endothelial inflammatory activation as well as to promote endothelial repair in vivo (Besler et al. 2011). Interestingly, the capacity to stimulate endothelial NO production could be improved upon exercise training (Adams et al. 2013).

HDL from patients with CAD and chronic heart failure (CHF) showed an elevated malondialdehyde (MDA) content as compared to HDL from healthy subjects, which may contribute to the impaired endothelial NO production (Besler

et al. 2011). The MDA-lysine adducts in HDL was determined to act via lectin-type oxidized LDL receptor 1 (LOX-1) activation of protein kinase C- β II, which blocks Akt-activating phosphorylation at Ser473 and eNOS-activating phosphorylation at Ser1177. Furthermore, inactivation of PON1 in HDL from healthy subjects resulted in attenuated NO production, in greater protein kinase C- β II activation, decreased activating eNOS-Ser1177 phosphorylation, and increased inactivating eNOS-Thr495 phosphorylation. Furthermore, HDL isolated from PON1-deficient mice failed to stimulate endothelial cell NO production (Besler et al. 2011). These observations likely suggest that alterations of HDL-associated PON1 may severely affect endothelial effects of HDL.

Very recently, we have also shown that MPO and PON1 reciprocally modulate each other's function in vivo (Huang et al. 2013). MPO may promote site-specific oxidative modification of PON1, therefore limiting its activity. In addition, HDL isolated from patients with an ACS carries enhanced chlorotyrosine content, site-specific PON1 methionine oxidation, and reduced PON1 activity (Huang et al. 2013).

Several groups have reported an inverse relationship between PON1 serum activity and cardiovascular events (Bhattacharyya et al. 2008; Regieli et al. 2009). Interestingly, analysis of SNPs for PON1 identified in genome wide association studies did not reveal a significant association between SNPs, associated with mildly reduced PON1 activity, and the risk of cardiovascular events (Tang et al. 2012). The interpretation of PON1 activity is difficult because it is not known to which extent the paraoxonase and arylesterase activities represent biologically relevant functions. Furthermore, we and others observed important post-translational modifications of the proteins, which could explain alterations of biological properties of the enzymes (Aviram et al. 1999; Besler et al. 2011; Huang et al. 2013).

5 Endothelial Anti-Inflammatory Effects of HDL

5.1 Mechanisms Under Physiological Conditions

Atherosclerosis is a chronic inflammatory disorder. The cellular inflammatory response is triggered by modified LDL accumulated in the subendothelium and the induction of proinflammatory cytokines and adhesion protein expression. As a consequence monocytes/macrophages infiltrate and accumulate in the arterial wall and express scavenger receptors and toll-like receptors to sequester cholesterol. The cholesterol-loaded macrophages together with T lymphocytes produce a wide array of proinflammatory cytokines and accelerate plaque progression (Hansson 2005).

In vitro, HDL has been shown to inhibit the expression of monocyte chemoattractant protein (MCP)-1, an important proinflammatory chemokine in endothelial cells (Mackness et al. 2004; Navab et al. 1991). Furthermore, the potential anti-inflammatory effects of HDL have been demonstrated by several in vivo studies. Infusion of native HDL as well as reconstituted HDL containing

apoA-I or apoA-I Milano suppressed cytokine and chemokine expression in animal models of inflammation (Calabresi et al. 1997; Cockerill et al. 1995). Reduced VCAM-1 expression and decreased monocyte/macrophage infiltration were reported after carotid artery cuff injury in apoE-deficient mice (Dimayuga et al. 1999). In the streptozotocin-induced diabetic cardiomyopathy rat model, human apoA-I gene transfer increased HDL-C plasma levels and blocked the diabetes-induced myocardial mRNA expression of VCAM-1 and ICAM-1 (Van Linthout et al. 2008). In contrast, overexpression of human apoA-I in apoE-deficient mice did not result in any change of endothelial VCAM-1 expression and monocyte adherence in early atherosclerotic lesions at the aortic branch sites, despite overall reduction in aortic atherosclerotic lesion formation (Dansky et al. 1999). These studies support the concept that the anti-inflammatory capacity of HDL is heterogeneous, and is dependent on the pathophysiological conditions. In vitro studies showed variations in the capacity of HDL isolated from different human subjects to reduce TNF- α stimulated endothelial VCAM-1 expression (Ashby et al. 1998; Van Lenten et al. 1995). In human studies, the administration of reconstituted HDL increased the anti-inflammatory capacity of HDL from patients with type-2 diabetes (Patel et al. 2009).

One of the proposed mechanisms for the anti-inflammatory effect of HDL is the removal of cholesterol from cell membranes of macrophages and also endothelial cells. The cholesterol depletion and consequently the lipid rafts disruption may downregulate signaling pathways and interfere with the antigen presentation or the expression of toll-like receptor (Anderson et al. 2000; Norata and Catapano 2012; Wang et al. 2012). Additional studies suggest that HDL and apoA-I are able to inhibit the capacity of antigen-presenting cells to stimulate T-cell activation. This inhibition was attributed to cholesterol efflux through ABCA1, an ATP-binding transporter (Tang et al. 2009; Zhu et al. 2010).

The anti-inflammatory capacity of HDL has been mainly attributed to apoA-I, the major protein constituent of HDL. In an in vivo study, apoA-I infusion in rabbits in acute vascular inflammation model reduced neutrophil infiltration and endothelial cell inflammatory activation (Puranik et al. 2008). In addition, apoA-I mimetic peptides have been shown to reduce vascular inflammation in type I diabetic rats and improve insulin sensitivity in obese mice (Peterson et al. 2007, 2008). It has also been demonstrated that treatment with lipid-free apoA-I and rHDL treatment reduced the expression of chemokines and chemokine receptors in vitro and in vivo by modulating NF- κ B and peroxisome proliferator-activated receptor γ (Bursill et al. 2010). Interestingly, apoA-I has also been shown to inhibit palmitate-induced NF- κ B activation by reducing Toll-like receptor-4 recruitment into lipid rafts (Cheng et al. 2012). More recently, De Nardo et al. using a systems biology approach identified the transcription factor ATF3 as an HDL-inducible gene to suppress Toll-like receptor-induced proinflammatory cytokines. Mice deficient in ATF3 and apoE revealed enhanced atherosclerotic lesions, and administration of rHDL into apoE-deficient mice resulted in ATF3 upregulation. The authors used rHDL in their study, suggesting that apoA-I is a key mechanistic player, and of

note, the effects seem to be unrelated to cholesterol transport (De Nardo et al. 2014).

The lipid moiety of HDL has also been proposed to be important for the anti-inflammatory effects of HDL. *In vitro* studies using reconstituted HDL containing only apoA-I with a few phospholipid molecules suggested that inhibitory effects of HDL on endothelial cell adhesion molecule expression are also, at least in part, dependent on HDL-associated phospholipid species (Baker et al. 2000). The inhibition of cytokine-induced expression of VCAM-1 by reconstituted HDL varied when different phosphatidylcholine species were used. This suggests that the lipid composition of HDL influences its anti-inflammatory capacity and is likely an important determinant of HDL functionality (Baker et al. 2000; Barter et al. 2004). In addition there are multiple reports suggesting the involvement of sphingosine-1-phosphate (S1P), a biologically active sphingolipid that plays key functions in the immune, inflammatory, and cardiovascular systems. S1P carried by HDL is believed to regulate arterial tone, vascular permeability, and tissue perfusion. During inflammation it induces endothelial adhesion molecules and recruits inflammatory cells, and furthermore it activates a negative feedback loop that consecutively decreases vascular leakage by improving the endothelial barrier function and preventing cytokine-induced leukocyte adhesion (Garcia et al. 2001; Lucke and Levkau 2010; Takeya et al. 2003).

5.2 Impaired Endothelial Anti-Inflammatory Effects of HDL in Patients with CAD, Diabetes, or Chronic Kidney Dysfunction

HDL isolated from patients with inflammatory disease such as CAD, diabetes, or chronic kidney disease display reduced eNOS activation and impaired endothelial repair capacity in a carotid artery injury mouse model. There have been various mechanisms proposed to account for the impaired endothelial anti-inflammatory effects of HDL. Reduced HDL apoA-I levels in inflammatory states has been related to accelerated HDL catabolism and apoA-I substitution in HDL particles by serum amyloid A (SAA) (Esteve et al. 2005; Khovidhunkit et al. 2004). In acute phase, SAA is able to replace apoA-I in HDL, resulting in reduced plasma levels of apoA-I (Parks and Rudel 1985). ApoA-I can be completely replaced by SAA in rabbits and mice, in a subset of small, dense HDL particles and therefore functioning as a structural apolipoprotein (Cabana et al. 1999). HDL isolated from patients with chronic kidney disease is enriched in SAA, which correlated with its reduced anti-inflammatory capacity to inhibit monocyte chemoattractant protein-1 formation in vascular smooth muscle cells (Tolle et al. 2012).

Moreover, HDL carries potential cardioprotective molecules that are significantly altered in compositions in patients with CAD or ACS (Riwanto et al. 2013; Vaisar et al. 2007). Oxidative modifications of HDL, in particular the amino acid residues in apoA-I, have been shown to contribute to the generation of dysfunctional HDL (Bergt et al. 2004; Shao et al. 2006, 2012; Zheng et al. 2004). *In vitro* study has demonstrated that MPO-catalyzed oxidative modification of HDL or

apoA-I converts HDL into a proinflammatory particle which promotes NF- κ B activation and endothelial VCAM-1 expression (Undurti et al. 2009). Furthermore, glycation of HDL and apoA-I, a process that is known to occur in diabetes in vivo (Curtiss and Witztum 1985), has been shown to impair the anti-inflammatory capacity of HDL (Nobecourt et al. 2010). Infusion of glycated lipid-free apoA-I failed to decrease adhesion molecule expression following vascular injury (Nobecourt et al. 2010). Moreover, glycation of HDL has also been shown to inhibit the HDL capacity to inhibit oxLDL-induced monocyte adhesion to human aortic endothelial cells in vitro (Hedrick et al. 2000).

6 Effects of HDL on Endothelial Cell Apoptotic Pathways

6.1 Mechanisms Under Physiological Conditions

Endothelial cell dysfunction and injury has been associated with the pathogenesis of atherosclerosis (Landmesser et al. 2004; Nabel and Braunwald 2012; Ross 1999). Studies in pigs have shown that atherosclerotic lesion-prone regions are characterized by a high endothelial cell turnover (Caplan and Schwartz 1973), which has been suggested to be due to increased endothelial cell apoptosis (Caplan and Schwartz 1973). Several studies have indicated an important contribution of endothelial cell apoptosis to the pathophysiology of CAD (Burke et al. 1997; Rossig et al. 2001). Furthermore, it is evident that apoptosis of endothelial cells and smooth muscle cells is detrimental to plaque stability (Bombeli et al. 1997; Kockx and Herman 2000). In the normal arterial wall, endothelial cells may protect themselves against apoptosis using NO-dependent mechanisms. The situation is completely different in atherosclerotic plaques, in a high oxidative stress environment where macrophages produce high amounts of nitric oxide or peroxynitrite that could induce apoptotic cell death (Kockx and Herman 2000). In the aorta the capacity of HDL to quench endothelial cell apoptosis may therefore represent an antiatherogenic property of HDL (de Souza et al. 2010; Nofer et al. 2001; Suc et al. 1997; Sugano et al. 2000).

HDL has been shown to attenuate endothelial cell apoptosis induced by different stimuli such as TNF- α , oxLDL, and growth factor deprivation (de Souza et al. 2010; Nofer et al. 2001; Suc et al. 1997; Sugano et al. 2000). It has been suggested that HDL may inhibit both death-receptor and mitochondrial-mediated apoptotic pathways. Importantly, both the protein and lipid moieties have been reported to contribute to the antiapoptotic capacity of HDL. ApoA-I has been shown to inhibit endothelial cell apoptosis induced by oxLDL, VLDL, and TNF- α (Speidel et al. 1990; Suc et al. 1997; Sugano et al. 2000). In a more detailed study, HDL subpopulations enriched with apoA-I account for approximately 70 % of the antiapoptotic activity of HDL in a cell culture model with human microvascular endothelial cells that were treated with mildly oxidized LDL. Using rHDL consisting of apoA-I, cholesterol and phospholipids potently inhibited oxLDL-induced apoptosis in these cells (de Souza et al. 2010). This suggests that apoA-I

plays an important role for the antiapoptotic capacity of HDL in oxLDL-stimulated endothelial cells.

HDL-associated lysosphingolipids have been identified to inhibit endothelial cell apoptosis induced by growth factor deprivation (Kimura et al. 2001, 2003; Nofer et al. 2001). The antiapoptotic capacity of the lipid moiety of HDL was further supported by the findings that the ratio of sphingosine-1-phosphate and sphingomyelin in small dense HDL3 particles was increased and correlated positively with the characteristics of these HDL subpopulations to inhibit endothelial cell apoptosis (Kontush et al. 2007).

Several mechanisms have been suggested for the endothelial antiapoptotic effects of HDL, depending on the stimulus of apoptosis. Endothelial cell death triggered by oxLDL causes a delayed but sustained increase in intracellular calcium which is shown to be prevented by HDL (Suc et al. 1997). HDL inhibits tumor necrosis factor- α -induced endothelial cell death by association as well as suppression of caspase-3, which is a key factor of all primary apoptotic pathways (Sugano et al. 2000). Nofer et al. suggested that sphingosine-1-phosphate (S1P) carried by HDL prevents the growth factor deprived activation of the intrinsic pathway to cell death (Nofer et al. 2001). Of note, lysophospholipids have been shown to enhance endothelial cell survival, but the effects were inhibited by S1P receptor knockdown, as well as by the presence of phosphoinositide 3 (PI3) kinase and Erk pathway antagonists (Kimura et al. 2003).

In addition, HDL activates Akt and causes phosphorylation of the Akt target Bcl-2-associated death promoter (BAD), preventing it from binding to the antiapoptotic protein Bcl-xL (Nofer et al. 2001). HDL also causes phosphoinositide 3 (PI3) kinase-mediated upregulation of the antiapoptotic Bcl-2 family protein Bcl-xL expression (Riwanto et al. 2013). Interestingly, HDL retained its antiapoptotic activity after knockdown of eNOS using specific RNA interference or pharmacological inhibition using L-NAME (Riwanto et al. 2013), indicating that HDL may exert its antiapoptotic activity independently of eNOS activation.

6.2 Impairment of the Endothelial Anti-Apoptotic Effects of HDL in Patients with Cardiovascular Disease

Recently, we reported that HDL isolated from patients with stable CAD or ACS, in contrast to HDL from healthy subjects, failed to inhibit endothelial cell apoptosis in vitro and in apoE-deficient-mice in vivo. Using proteomic analysis of HDL, we observed reduced clusterin and increased apo C-III content in HDL isolated from patients with CAD. Both, supplementing HDL from healthy subjects with apoC-III or pretreatment of healthy HDL with an antibody against clusterin, lead to the activation of proapoptotic signaling pathways in endothelial cells (Riwanto et al. 2013). HDL isolated from these patients stimulated endothelial proapoptotic pathways, in particular p38-MAPK-mediated activation of the proapoptotic Bcl-2-protein tBid. Our study further suggests that differences in the proteome of HDL from patients with CAD, in particular reduced HDL-associated clusterin and increased HDL-associated apoC-III, play an important role for altered activation

of endothelial anti- and proapoptotic signaling pathways (Riwanto et al. 2013). In line with this, a nested case-control study found that cholesterol levels in apoC-III-containing HDL particles show a positive association with cardiovascular risk, whereas both total HDL-C and cholesterol in apoC-III-free HDL particles show the expected inverse associations (Jensen et al. 2012). In addition, Undurti et al. demonstrated that MPO-catalyzed oxidation of HDL impaired its capacity to inhibit endothelial apoptosis in vitro (Undurti et al. 2009).

7 HDL Protein Cargo and Prognostic Biomarkers

As highlighted above, alterations in HDL functions are likely, at least in part, attributed to changes in the composition of HDL particles. HDL particles are highly heterogeneous in their structure and compositions. They undergo continuous remodeling through apolipoprotein exchanges with other circulating lipoproteins and tissues (Lund-Katz et al. 2003; Saito et al. 2004).

An early study has shown that acute phase response causes significant changes in the HDL-associated proteins and apolipoprotein composition, i.e., enrichment in C-reactive protein (CRP), secretory phospholipase A₂-IIa (sPLA₂-IIa), serum amyloid A (SAA), and cholesterol ester transfer protein (CETP) (Coetzee et al. 1986). It has been suggested that such remodeling may compromise the antiatherogenic functions of HDL (Jahangiri et al. 2009). These observations have prompted explorative studies into HDL composition. Evaluations of the HDL proteome have been reported with one- or two-dimensional electrophoresis combined with high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) or matrix-assisted laser desorption/ionization-time flight mass spectrometry (MALDI-TOF-MS) (Heller et al. 2007; Karlsson et al. 2005). More recent studies have used shotgun proteomics, through label-free MS techniques, such as spectral counting and XIC inspection, to analyze HDL protein compositions (Riwanto et al. 2013; Vaisar et al. 2007). Overall, the identification techniques currently available for the analysis of the HDL proteome are semi-quantitative.

Given the complexity of HDL and its multitude of biological functions, it is conceivable to expect that sets of functionally associated proteins can provide information about their participation in the spectrum of atheroprotective actions attributed to HDL. This has further led to studies comparing the HDL proteome of healthy subjects with that of particles from patients with dyslipidemias or CAD (Green et al. 2008; Heller et al. 2007; Riwanto et al. 2013; Vaisar et al. 2007). In the study by Vaisar et al., HDL was shown to carry apolipoproteins and proteins with functions in lipid metabolism, the acute phase response, complement regulation, and blood coagulation (Vaisar et al. 2007). The same group further compared the proteome of HDL3 from 6 CAD patients before and after 1-year treatment with combined statin/niacin therapy (Green et al. 2008). In this study, HDL3 of CAD patients is significantly enriched in apoE and apoCII and carry less apoJ and phospholipid transport protein (PLTP) as compared to HDL from control subjects. Treatment with niacin/statin decreases HDL3 apoE and raises apoJ and PLTP levels

(Green et al. 2008). In our recent study, we have observed that reduced clusterin and increased apoC-III content in HDL isolated from patients with CAD lead to activation of proapoptotic signaling pathways in endothelial cells (Riwanto et al. 2013).

Of note, oxidative modifications of HDL-associated proteins have also been demonstrated to contribute to the functional impairment of HDL. In particular, MPO-mediated oxidation of apoA-I has been linked to the generation of dysfunctional forms of HDL (Huang et al. 2014; Shao et al. 2010; Undurti et al. 2009; Wang et al. 2007b; Zheng et al. 2004). Recently, we have shown that MPO and PON1 interact and reciprocally modulate each other's function *in vivo*, i.e., PON1 partially inhibits MPO activity, while MPO oxidizes and inactivates PON1 (Huang et al. 2013). Furthermore, HDL isolated from patients with an ACS carries enhanced chlorotyrosine content, site-specific PON1 methionine oxidation, and reduced PON1 activity (Huang et al. 2013).

Taken together, these observations indicate that the HDL proteomic composition may be altered in different disease states which likely have an impact on HDL function. Unraveling the complexities of the HDL through proteomics studies may therefore allow for better understanding of the HDL composition and function. However, proteomics studies of HDL have been limited in size and number due to the labor-intensive nature of the approach. Furthermore, given the diverse concentration range of the proteins in the HDL particles, some proteomics approaches may fail to reflect the actual amount or alteration of low abundant proteins due to signal suppression in the presence of highly abundant proteins. Targeted proteomics using selected reaction monitoring is a powerful tandem mass spectrometry method that can be used to monitor target peptides within a complex protein digest (Picotti and Aebersold 2012; Picotti et al. 2010). The approach will enable absolute quantification of the individual protein contained within HDL particles.

Conclusion and Perspectives

Over the past two decades, many studies have emerged showing various antiatherogenic effects of HDL, including the capacity of HDL to mediate reverse cholesterol transport, and antioxidative, anti-inflammatory, and antiapoptotic effects (Riwanto and Landmesser 2013). Growing evidence has shown that HDL particles are highly heterogeneous and the vasoprotective effects of HDL are altered in patients with CAD, diabetes, and chronic kidney dysfunction, i.e., patients with a high cardiovascular risk profile. Of note, these findings have more recently been supported by observations in cohort studies, suggesting that in patients with advanced CAD, higher plasma levels of HDL-C are no longer associated with reduced risk of cardiovascular events (Angeloni et al. 2013; Schwartz et al. 2012). Importantly, the increase in HDL-C observed with some lipid-modifying drugs has not been uniformly associated with clinical benefit (Barter et al. 2007; Schwartz et al. 2012). These findings have clearly indicated that the association between HDL and cardiovascular disease is far more complex than previously thought and is likely mediated by different HDL

functional properties, independent of the cholesterol component of the HDL particle (Heinecke 2011).

It is increasingly evident that the functional heterogeneity of HDL may be attributed to the complexity of the HDL particles, containing proteins and lipids that can be modified or altered in their composition. Of note, rapid advances in the MS proteome technology have allowed tremendous leaps in characterizing the HDL proteome. Studies investigating the lipidome of HDL are still somewhat limited by the available technologies. Importantly, several studies have associated changes in specific HDL protein either due to altered composition or modification to the loss of function of HDL (Besler et al. 2011; Huang et al. 2013; Riwanto et al. 2013; Shao et al. 2006, 2012; Zheng et al. 2004). Nevertheless, it remains to be determined if the alterations are secondary to changes that occur during the disease progression or if the alteration has indeed a causal effect on disease etiology. In this context it is also important to emphasize the problem of confounding between different diseases: for example, many diabetic patients have (sub)clinical chronic kidney disease and/or CAD. As a consequence one must also expect considerable overlap of HDL modifications which were initially identified in patients with either diabetes, CAD, or CKD. It is hence rather unlikely that each disease has its specific footprint of HDL dysfunction and alterations in proteome and lipidome.

Taken together, it is becoming clear that plasma HDL-C levels are not an appropriate marker of vascular effects of high-density lipoproteins and therefore do not represent a reliable therapeutic target. Importantly, HDL-targeted treatment approaches need to take into account the altered vascular effects of HDL in cardiovascular disease. Therefore, targeting HDL-mediated antiatherogenic mechanisms, rather than plasma HDL-C levels, may represent a more promising and interesting therapeutic target. Studies looking into the specific mechanisms leading to a loss of antiatherogenic effects of HDL are of particular importance, in order to develop therapeutic approaches that aim to restore the vasoprotective properties of HDL.

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